A Pre-Synopsis entitled as

"Exploration of *Bacillus spp*. For Microbiological Control of Stem rot Disease of Groundnut."

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1.INTRODUCTION

Groundnut crop is considered as most important crop in the World (13th) and is most important source of edible oil (4th). Similarly, groundnut is ranked as 3rd most important source of vegetable protein in the world (Smith 2002). Groundnut is one of the widely cultivated and produced oilseed crop in World. It is cultivated in more than 100 countries of the world and that's why it is referred as a "Universal Crop". Groundnut is called as the 'king' of oilseeds. It is one of the most important food and cash crops of our country. While being a valuable source of all the nutrients, it is a low-priced commodity. Groundnut is also called as wonder nut and poor man's cashew nut.

Groundnut is grown on a large scale in almost all tropical and subtropical countries of the world as this type of weather conditions suit well. It is estimated that around 65% of the crop produced in the world is crushed to extract groundnut oil and the rest is used in making other edible products. The most important groundnut growing countries are India, China, Nigeria, Sudan, and USA (Handbook on Grading of Food Grains and Oilseeds). As of 2019, China was the largest producer of Groundnuts worldwide, holding an estimated 36 % share of global production. India ranked second that year, producing about 14 % of the world's Groundnut. India occupies the first place in acreage and second in production after China. (https://www.statista.com/statistics/1030846/major-producers-of-peanut-worldwide)

Groundnut is the largest oilseed in India in terms of production. At the end of Year 2019, India produced more than six million metric tons of groundnut. This was a decrease from the previous year. Among the survey of states carried out by Agriculture& Processed Food Products Export Development Authority (Ministry of Commerce &Industry, Government of India). The highest yield of 2051 kg/ha was estimated for Rajasthan, followed by 1421 kg/ha for Gujarat, 1361 kg/ha for Maharashtra, 883kg/ha for Andhra Pradesh and 750 kg/ha for Karnataka. The national average yield was estimated at 1336 kg/ha. The combined production of these five states wise estimated at 43,47,298 MT which accounted for 83.6% of the estimated national production. With 20,84,780 MT, Gujarat contributed 40.1% of the national production followed by Rajasthan (11,26,206 MT; 21.6%.) Andhra Pradesh (5,82,972; 11.2%),Karnataka (2,87,178 MT; 5.5%) and Maharashtra (2,66,162; 5.12%) while the joint contribution of the remaining states was estimated at 8,48,698 MT i.e. 16.4%. Thus, the all-India kharif 2018 production was estimated at 51,95,990 MT. (http://apeda.gov.in/apedawebsite/SubHead_Products/Ground_Nut.htm).

Groundnut contains on an average 40.1 per cent of fat and 25.3 percent of protein and is a rich source of calcium, iron and vitamin B complex like thiamine, riboflavin, niacin, and vitamin A. It has multifarious usages: It is used not only as a major cooking medium for various food items but also for manufacture of soaps, cosmetics, shaving creams and lubricants.

2.Review of literature and Development in subject:

Sclerotium rolfsii, a broad host range fungus, caused Stem rot, the major soilborne disease of groundnut (*Arachis hypogaea*). In India among the soil-borne fungal diseases of groundnut, stem rot caused by *S. rolfsii* is a potential threat for groundnut grown under irrigated conditions.

Stem-rot caused by *S. rolfsii* is sporadic in most of the groundnut growing areas of the India and is assuming importance in Tamil Nadu, Andhra Pradesh, Karnataka and Maharashtra (Pande and Narayana Rao, 2000). The disease epidemics have also been reported from Madhya Pradesh and Gujarat. Groundnut pod yield losses due to *S. rolfsii* in Maharashtra were estimated to the tune of 25 % (Mayee and Datar, 1989). Stem-rot caused

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up to 27 % loss in Uttar Pradesh and in the Deccan Plateau (Pande and Narayana Rao, 2000). Approximately 5-15 % loss in the initial crop stand is due to seed-rot and seedling collapse (Pande and Narayana Rao, 2000). Additionally, pod deterioration caused by the soil- borne pathogenic fungi has been reported to be potentially serious in several farmers fields in Andhra Pradesh, Tamil Nadu and Karnataka (Pande and Narayana Rao, 2000). *S. rolfsii* is a plurivorous fungus infects more than 500 species of plants belonging to about 100 families and turns economically important disease in warm, moist climate throughout the world (Aycock, 1966; Punja, 1985; Sarma *et al*., 2002 and Azhar *et al*., 2003).

S. rolfsii is especially severe on legumes, Solanaceous crops, cucurbits and other vegetables grown in rotation with beans (Hall, 1991). The fungus affects nearly 500 plant species comprising Composite and Leguminosae. Graminaceous species are less susceptible (Mehan *et al*., 1995).

Stem rot disease management can be carried out by using chemical fungicides as well as cultural practices. Some of the cultural practices which can be adopted easily by farmers are:

1. Adjustment of the date of sowing if possible so that the susceptible stage of the crop growth does not coincide with the highly favourable weather for pathogens to establish and cause greater damage to groundnut crop.
2. Removal of infected debris from field and its destruction by burning which is recommended for control of foliar diseases.
3. Crop rotation with non-host crops can reduce the incidence of soil- borne diseases, but it is not a practical proposition under rainfed cultivation of groundnut in India.

While chemical practices used by farmers are:

1. A mixture of fungicides, viz. terrachlor + terrazole @ kg ha-1 + 40 kg ha- 1 at pegging was found effective in controlling stem-rot disease.
2. Pentachloronitrobenzene (PCNB), Carboxin, and PCNB in combination with ethoprop were the only pesticides recommended for the control of stem rot (Dwivedi and Prasad, 2016).
3. When compared with older compounds, new compounds, including tebuconazole, flutolanil and diniconazole, reduced stem rot occurrence by more than 50 %; yield increases of 224 kg/ha or more were associated (Hagan *et al*., 1991).

Yet despite continuous research on *S. rolfsii* over last 118 years since reported first by Rolfs in 1892 on tomato, this pathogen continues to plague growers and cause considerable economic losses. Disease control efforts have often met with limited success, due to the extensive host range, prolific growth, and ability to produce large numbers of sclerotia that persist in soil for several years and responsible for initiation of primary infection (Aycock, 1966, Punja 1985).

Increasing incidence of stem rot of groundnut caused by *S. rolfsii* observed in Marathwada region and its danger towards groundnut growers, it was felt necessary that stem rot severity can be reduced with the help of biological control agents as an alternative eco-friendly measure.

The traditional agricultural practice to control the phytopathogen *S. rolfsii* is by using variety of fungicides e.g. Bavistin, Captan etc. But a severe disadvantage of the traditional method is that it is not effective to check the *Sclerotium* during the cropping period (90- 100 days) and is not eco-friendly. Because of the increased usage of chemical fungicides,

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produced concern for the environment and human health, microbial inoculants have been experimented extensively during the last decade to control wilt and other plant diseases (Siddiqui and Shakeel, 2006; Chakraborty and Chatterjee, 2008; Akhtar *et al.*, 2010).

One of the most promising alternatives to synthetic fungicides is biological control of pathogens, which includes the use of biofungicides based on antagonistic microorganisms. In contrast to commonly used fungicides, biofungicides have several advantages: high specificity against target pathogens, rapid degradation in the environment and low mass-production cost. Antagonistic microorganisms operate through various modes of activity such as competition with pathogens for space and nutrients, production of antibiotics and cell-wall degrading enzymes and reduction of pathogen population by hyperparasitism (Živković *et al.*, 2010, Stanojević *et al.*, 2016).

Most bacterial strains commercially used as biofungicides belong to the genera *Bacillus* and *Pseudomonas* (Fravel, 2005). *Bacillus spp.* produces spores that are resistant to various physical and chemical treatments such as heat, desiccation, UV irradiation and organic solvents (Leelasuphakul *et al.*, 2008). Also, they are known to produce an array of secondary metabolites, including antibiotics, cell-wall degrading enzymes and antifungal volatile substances. This indicates that *Bacillus spp.* strains can be efficient biological control agents against a wide range of plant pathogens (Kim and Chung, 2004; Leelasuphakul *et al.*, 2006). Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens (Haas and Defago, 2005; Stein, 2005; Ge *et al.*, 2007). For instance, *Bacillus subtilis* produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturins, surfactins and zwittermycin (Asaka and Shoda, 1996; Stein, 2005) and it secretes also hydrolytic enzymes, i.e. protease, glucanase (Cazorla *et al.*, 2007), chitinase (Manjula *et al.*, 2004), lipase (Detry *et al.*, 2006) and amylase (Konsoula and Liakopoulou-Kyriakides, 2006).

Microbiological control against *Sclerotium rolfsii* **Sacc:**

A new approach has been emerging to tackle these effects produced using fungicides. This approach is popularly known as microbiological control of phytopathogen. Several investigators have described the inhibition of growth and sclerotial production in *S. rolfsii* by Bacteria (Rangeshwaran *et al.*, 2002; Abd-El-Moity *et al.*, 2003).

Although several bacteria species are known for their antifungal activity, *Bacillus spp.* is one of the promising antifungal-producing bacteria because it can form endospore also can tolerate extreme pH, temperature, and osmotic condition. Therefore, from an ecological perspective, their application is sustainable (long term) (Ashwini and Srividya, 2014). Moreover, *Bacillus spp.* regarded as safe biological agents and their potential is considered high, due to diverse range modes of actions including antibiosis, production of siderophores, cell wall degrading enzymes, and lipopeptides producer (Islam *et al.*, 2012, Reynaldi, *et al.*, 2016). *Bacillus subtilis*, one of most well studied *Bacillus species*, is known to have an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). Among these antimicrobial compounds, cyclic lipopeptides of the surfactin, iturin, and fengycin families have been well recognized for their function and potential uses in biotechnology application (Ongena and Jacques, 2007).

To discover an eco-friendly microbial control alternative to these fungicides, by applying bacteria specially *Bacillus sp.* as a control measure of this fungal phytopathogen. *Bacillus sp.* has been searched as biocontrol because of (i) it is among the most prominent bacteria found to colonize plants root and soil populations (ii) it forms endospore (iii) it causes direct antagonism on the pathogen, as well as, by induction of host resistance and (iv) It largely produce structurally diverse antibiotics, produce of iron chelators, to cause antagonism of phytopathogen (Shafi, *et al.*, 2017).

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Similarly, other authors also found *Bacillus spp.* effective microbiological control agents against *Sclerotium rolfsii*, such as:

Preliminary studies conducted at the Viticultural and Oenological Research Institute (YORI) Republic of South Africa, indicated that *Bacillus subtilis*, isolated from vineyard soil, had an inhibitory effect on the mycelium growth of *S. rolfsii* in vitro. Wong and Hughes (1986) isolated a *Bacillus spp.* from soil antagonistic to *S. cepivorum* on onion. *B. subtilis* is a well-known fungal antagonist and has been proven by several workers to be a successful biological control agent against different fungal diseases (Baker *et al.*, 1983, Singh and Deverall, 1984, Baker and Stavely, 1985, Keyser and Ferreira, 1988).

Bacillus subtilis was tested Kobayashi *et al.* (2000) for its antifungal activity against *Sclerotium rolfsii.* Antifungal activity was demonstrated by the formation of zone of inhibition around the spots of *Bacillus subtilis.* It was observed that in varying incubation time the antifungal activity was also varied in the form of zone of inhibition. Patil *et al.*, (2001) recorded inhibition of *Sclerotium rolfsii* by *Bacillus* sp. 102 (1 mg. 50 ml-1) which showed hyphal tip bursting in all the test fungi inhibited 70 percent growth of S. *rolfsii.* Similar findings were also observed by the study conducted by Souto *et al.* (2004) where mycelia growth of *Sclerotium* spp. was inhibited by application of *Bacillus* spp. using the dual culture technique.

Formerly, several pest management programs have successfully incorporated the application of some *B. subtilis* strains (Jacobsen *et al.*,2004). Nalisha *et al.*, (2006) reported that antifungal compound produced by *B. subtilis* could inhibit 58.3% of *S. rolfsii* growth. Figueredo *et al.*, (2014) reported that *Bacillus sp. CHEP5* was a biocontrol agent that induces systemic resistance (ISR) in *Arachis hypogaea* L. (peanut) against *Sclerotium rolfsii*, the causal agent of root and stem wilt.

Furthermore, Shifa *et al.*, (2015) showed that *B. subtilis* G-1 significantly controlled stem rot disease of groundnut and increased the plant growth under greenhouse conditions.

3.Objectives of the research:

Considering the importance of Groundnut crop and the influence of *Sclerotium rolfsii* on productivity in Marathwada region, following objectives were set to explore the problem and study it in detail to find out bio management strategies for control of Sclerotium rolfsii, and to improve the yield of Groundnut crop in a cost-effective and ecofriendly manner.

- Isolation of stem rot pathogen from infected Groundnut crop cultivated in Marathwada region and its pathogenicity testing.
- Isolation of *Bacillus spp.* from rhizospheric soil and its screening for inhibition of *Sclerotium rolfsii* by *in vitro* method.
- Identification of the efficient Antagonistic *Bacillus spp.*
- Illustration of biocontrol mechanism of efficient Antagonistic *Bacillus spp.* against *Sclerotium rolfsii.*
- Illustration of Growth Promotion Ability of Efficient Antagonistic *Bacillus spp.*
- Preparation of a carrier-based user-friendly formulation of efficient *Bacillus spp.*
- Confirmation biocontrol ability of selected *Bacillus spp.* by Pot assay.
- Exploration of growth promoting ability of efficient *Bacillus spp.* by pot assay.

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3.1 Hypotheses:

Traditionally farmers use fungicides as well bactericides to protect the crop from diseases. Chemical control is one of the promising strategies for management of phytopathogens of crops. However, use of fungicides and bactericides cause environmental pollution. The proposed hypothesis is mainly targeted on the control of plant diseases using naturally occurring antagonistic microorganisms, such as *Bacillus spp.* This approach is popularly known as biocontrol of plant pathogens.

Biocontrol is a bio-based, eco-friendly strategy, offers a practical and economical alternative for the management of plant pathogens with a potential to emerge as an alternative to chemical control. Due to these facts about the management of Phytopathogen, there is need to search for eco-friendly, target specific alternative for the management of Stem Rot Disease of Groundnut.

4. Methodology to be adopted:

To confront with this problem, following methodology will be adopted.

1. Isolation of Stem Rot pathogen from infected Groundnut crop cultivated in Marathwada region and its pathogenicity testing:

For isolation of Phytopathogen, infected Groundnut crop will be collected from different fields in Marathwada region and brought to laboratory in polythene bags. Isolation of Phytopathogens will be carried out by method used by (Rakh, 2010) from infected stem. Pathogenicity test will be carried out to prove the Koch's postulates. Fungal phytopathogens will be identified by using the book Subramanian (1971).

2. Isolation of *Bacillus spp.* from rhizospheric soil and its screening for inhibition of *Sclerotium rolfsii* by In Vitro method.

For isolation of rhizospheric *Bacillus spp.*, rhizospheric soils from different fields of Marathwada Region will be collected in polythene bags and brought to laboratory. Isolation of *Bacillus spp.* will be carried out by using method followed by Liu et al., (2020) and its screening for efficient biocontrol will be done by method of Rakh *et al.*, (2020)

3. Identification of efficient *Bacillus spp.*:

Identification of efficient *Bacillus spp.* will be carried out by morphological cultural biochemical characteristics and 16 S rRNA Sequencing.

4. Illustration of Biocontrol Mechanism of Efficient *Bacillus spp.*:

To illustrate the biocontrol mechanism, the efficient *Bacillus spp.* will be tested to produce Non-volatile diffusible antibiotic, volatile metabolite, and siderophore and cell wall degrading enzymes (Kotasthane *et al.*, 2015).

5.Illustration of Growth Promotion Ability of Efficient Antagonistic *Bacillus spp.*:

To illustrate the biocontrol mechanism, the efficient *Bacillus spp.* will be tested to phosphate solubilizing activity (Dipak *et al.*, 2016) and produce growth promoting hormone. (Ewa *et al.*, 2020)

6. Preparation of formulation for easy use of biocontrol agents:

Carrier based powder formulation (CBPF) of microbial control agents will be prepared and used for further study.

7. Pot Assay for microbial control

By using the Pot Assay, the biocontrol ability of selected *Bacillus spp.* will be carried out according to Yaqub and Shahzad (2005).

8. Growth Promotion ability:

Growth promoting ability of *Bacillus spp.* will be analyzed by direct inoculation in Pot and studying different parameters.

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5.Importance of Study :

5.1 International Status: -

Internationally, several investigators have reported the inhibition of growth and *sclerotial* production in *S. rolfsii* by bacteria as follows:

Dey *et al.*, (2004) studied nine different isolates of PGPR from a pool of 233 rhizobacterial isolates from groundnut rhizosphere on the basis of ACC-deaminase activity. These nine isolates were selected, initially, on the basis of germinating seed bioassay in which the root length of the seedling was enhanced significantly over the untreated control. All the nine isolates were identified as *Pseudomonas* spp. Four of these isolates, viz. PGPR1, PGPR2, PGPR4 and PGPR7 (all fluorescent pseudomonas), were the best in producing siderophore and indole acetic acid (IAA). In addition, *Pseudomonas fluorescens*PGPR1 also possessed the characters like tri-calcium phosphate solubilization, ammonification and inhibited *Aspergillus niger* and *A. flavus in vitro* while PGPR4 showed strong *in vitro* inhibition to *Sclerotium rolfsii.* Seed bacterization with plant growth-promoting *P. fluorescens* isolates, viz. PGPR1, PGPR2 and PGPR4, suppressed the soil-borne fungal diseases like collar rot of peanut caused by *A. niger* and PGPR4 also suppressed stem rot caused by *S. rolfsii.*

(Abd *et al.*,2007) formulated *Bacillus subtilis* for groundnut seed (*Arachis hypogaea cv.shulamit*) counteracted the destructive effect of the seedborne pathogen *sclerotium* (Athelia) *rolfsii* on the nodulation , leghemoglobin and nitrogenase activity of groundnut. Also, the changes in crop vigor index, total nitrogen content and survivability of both *Rhizobium spp.* and *Bacillus subtilis* have been related to compatibility and even an occasional synergism between them.

Xu *et al.*, (2020) isolated the bacterial strain LX11 from the groundnut (*Arachis hypogaea*) rhizosphere was identified as *Bacillus pumilus* drew on its phenotypic features, 16S ribosomal RNA and β-subunit of DNA gyrase (gyrB) sequence. LX11 exhibited antimicrobial activities against *S. rolfsii.* The action of LX11 against *S. rolfsii* was shown to gain 65% in field examination. LX11 may be useful as a capable biocontrol strain for southern blight of groundnut.

5.2 National Status:-

In India, the national institute actively involved in the management of stem rot diseases of groundnut crop. Research Institute and Several investigators have reported inhibition of *S. rolfsii,* few of them are as follows.

Abdel *et al.*, (2018) isolated four of *Bacillus subtilis* named, B4, B7, B8 and B10 and tested for their biocontrol capability and antagonistic effect on the vitro growth of certain phytopathogenic fungi of groundnut, *Rhizoctonia solani* and *Sclerotium rolfsii. Bacillus subtilis* isolate B4 was the dreadfully effective one for inhibiting the fungal mycelia growth. And also, *Bacillus subtilis* isolate B4 was formulated and evaluated as biocontrol to lower groundnut soil borne disease under green house and field condition at the side of Rizolex –T (fungicide) as standard. Similarly the formulation of *B. subtilis* and Rizolex –T in a soil infested with *R. solani*, *S . rolfsii* and mixture of them were more effectual in decreasing percentage of damping off, root and pod rot disease incidence (%) in greenhouse and open field environment during the two season 2015 and 2016. Formulated PGPR *Bacillus subtilis* B4 gave higher increasing yield percentage than treatment by Rizolex –T in two evaluated

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season 2015 and 2016. These result shows that bio formulated agent was more efficient as fungicide when contrast with the other chemical synthesised fungicides, safe for human and the environment and economy.

Puja *et al.*, (2021) in this study pointed that the rhizospheric bacterial isolate showed antagonistic property against soil-borne fungal pathogen. Five rhizospheric *Bacillus* species viz. *Bacillus subsp. substilis str.*168, *B. siamensis* strain PDA 10, *B. amyloliquefaciens* strain 1034, *B. velezensis* strain FZB42165, and *B. atrophaeus* strain NBRC15539 were assessed for their antagonistic potential against *S rolfsii* based on 3 different screening methods. Among these 100% fungal growth inhibition was observed by all 5 *Bacillus spp.* in novel ring method. Whereas in dual culture method the maximum growth inhibition was (58%) exhibited by strain NBRC 15539 of *B. atrophaeus*. The antagonistic activity showed by the modified dual culture method was also comparatively high, and highest activity (93.7 %) was shown by strain NBRC 15539 of *B. atrophaeus.* Besides, the *Bacillus spp.* was also assessed for their plant growth –promoting attribute and other capabilities such as the production of siderophore, HCN, amylase, protease, lipase, and ammonia, counting their assessment for chitinase and cellulase activity.

Swaroopa and Madhuri *(2021)* isolated *Bacillus zhangzouensis*, *Bacillus velezensis* from rhizospheric soil which shown highest inhibition of *Sclerotium rolfsii* in dual culture method while in field results reduced 60% incidence of stem rot disease of groundnut. These cultures in mixture showed growth promotion ability also. But when used individually, either of the *Bacillus spp.* could not promote the plant growth to the extent they can promote when used as mixed culture

Gurikar *et al.*, (2022) reported that *Bacillus subtilis* is potential plant growth stimulating and biocontrol bacterium that can be utilise to manage the plant disease. *bacillus* species have multiple mechanism, viz., antibiosis, competition (nutrient). Mycoparasitism, and induced systemic resistance in host plants, which make it an ideal biocontrol agent.

All these authors reporting suggest the usefulness of *Bacillus spp.* as biocontrol and growth promoter component in the integrated management of groundnut stem rot. Still there is need to find effective biocontrol measures in the form of *Bacillus spp.* against *Sclerotium rolfsii*, a stem rot causing soil borne pathogen of groundnut and formulate it. Groundnut being a major cash crop, hence there is crucial need to search for an efficient biocontrol agent instead of chemical fungicide which can be formulated in a manner easy to be used by the farming community and will effectively control soil born phytopathogen.

5.3 Significance of Study:

To improve the yield of groundnut both quality and quantity wise and to satisfy the demands of ever-increasing population. In this context variety of synthetic agrochemicals are used by farmers to control the Phytopathogens attacking the crop. This practices has led to many environmental crises like,

i. Disturbance of ecological balance of soil.
ii. Contamination of ground water.
iii. Development of resistance among the pathogens towards the synthetic chemicals.
iv. Sever health risk to non target species like humans.

So, there is need of permanent and assured solution to the problem of applying synthetic agrochemicals in agriculture for control of the phytopathogens by farmers. To cope up with

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this problem an attempt will be made through this research work by using a target specific, rhizospheric microorganisms for efficient control of phytopathogen causing Stem rot disease to groundnut, in an eco-friendly and cost effective manner.

Significance of this study lies in the fact that the groundnut growers can get an alternative for management of stem rot phytopathogen. Microbial control agents along with control of diseases also, well known to promote the growth of plants without additional application of fertilizers. Hence fertilizer input can also be reduced.

6.Proposed work plan (Time schedule of activity):

Sr. no.	Duration(Months)	Work to be done
1	6 month	Isolation of stem rot pathogen from infected Groundnut crop cultivated in Marathwada region and its pathogenicity testing.
2	12 months	Isolation of *Bacillus spp.* and its screening for inhibition of *Sclerotium rolfsii* by In Vitro method.
3	18 months	Identification of efficient *Bacillus spp.*
4	24 months	Illustration of Biocontrol Mechanism of Efficient *Bacillus spp.*
5	30 months	Illustration of Growth Promotion Ability of Efficient Antagonistic *Bacillus spp.* Preparation of formulation for easy use of biocontrol agents.
6	36 months	Pot Assay for microbial control & Growth Promotion ability

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